Fatty Acid Export from the Chloroplast. Molecular Characterization of a Major Plastidial Acyl-Coenzyme A Synthetase from Arabidopsis[1]

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Acyl-coenzyme A (CoA) synthetases (ACSs, EC 6.2.1.3) catalyze the formation of fatty acyl-CoAs from free fatty acid, ATP, and CoA. Essentially all de novo fatty acid synthesis occurs in the plastid. Fatty acids destined for membrane glycerolipid and triacylglycerol synthesis in the endoplasmic reticulum must be first activated to acyl-CoAs via an ACS. Within a family of nine ACS genes from Arabidopsis, we identified a chloroplast isoform, *LACS9*. *LACS9* is highly expressed in developing seeds and young rosette leaves. Both in vitro chloroplast import assays and transient expression of a green fluorescent protein fusion indicated that the LACS9 protein is localized in the plastid envelope. A T-DNA knockout mutant (*lacs9-1*) was identified by reverse genetics and these mutant plants were indistinguishable from wild type in growth and appearance. Analysis of leaf lipids provided no evidence for compromised export of acyl groups from chloroplasts. However, direct assays demonstrated that *lacs9-1* plants contained only 10% of the chloroplast long-chain ACS activity found for wild type. The residual long-chain ACS activity in mutant chloroplasts was comparable with calculated rates of fatty acid synthesis. Although another isozyme contributes to the activation of fatty acids during their export from the chloroplast, LACS9 is a major chloroplast ACS.

Lipid metabolism plays essential roles in normal plant growth and development and is a complex, highly regulated process. Lipids can constitute a significant proportion of plant tissues. In oilseeds, up to 60% of the dry weight can be accounted for by lipids in the form of triacylglycerol (Browse and Somerville, 1994; Ohlrogge and Jaworski, 1997). In vegetative plant cells, lipids account for 5% to 10% of dry weight, mostly as glycerolipids and other components of membranes (Ohlrogge and Browse, 1995). Fatty acids are components of glycerolipids and also precursors for the synthesis of cutin and epicuticular wax that provide an outer barrier against environmental and biological stresses. The hormone jasmonic acid is also synthesized from a fatty acid precursor. Because the products of fatty acid metabolism are involved in virtually every aspect of cellular biochemistry, considerable effort has gone into elucidating the pathways of fatty acid and lipid metabolism. The major pathways for membrane lipid and triacylglycerol synthesis are well understood, but various details remain unknown. One detail that is not well characterized is the role of acyl-CoA synthetases (ACSs) in fatty acid synthesis. ACSs (EC 6.2.1.3) catalyze the formation of a thioester compound from free fatty acid, ATP, and CoA (Kornberg and Pricer, 1953). The reaction proceeds through a two-step mechanism involving the conversion of free fatty acid and ATP to an enzyme-bound acyl-AMP intermediate in the presence of Mg^{2+}(reaction 1). Next, the thioester bond formation with CoA generates free AMP and acyl-CoA (reaction 2).

$$\text{Fatty acid} + \text{ATP} \leftrightarrow \text{fatty acyl-AMP} + \text{inorganic pyrophosphate} \quad (1)$$

$$\text{Fatty acyl-AMP} + \text{CoA} \rightarrow \text{fatty acyl-CoA} + \text{AMP} \quad (2)$$

Fatty acyl-CoAs produced by the ACS reaction participate in many aspects of plant metabolism (Ichihara et al., 1997; Gargiulo et al., 1999), including both the synthesis of glycerolipids and other fatty acid derivatives, and the breakdown of lipid reserves via β-oxidation (Gerhardt, 1992). Therefore, it is important to learn more about ACS enzymes in plants.

To date, our knowledge of the contribution of ACSs to lipid metabolism has been based on genetic and biochemical studies in *Escherichia coli* and yeast (*Saccharomyces cerevisiae*). In these organisms, ACSs are involved in the transport and activation of fatty acids (DiRusso and Black, 1999). In *E. coli*, exogenous long-chain fatty acid transport and activation requires the action of *FadL* and *FadD*, which encode a protein that

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binds fatty acids and transfers them across the outer membrane and an inner membrane-associated ACS that activates the fatty acids, respectively (Black et al., 1992). In yeast, long-chain fatty acid transport requires Fat1p (DiRusso and Black, 1999). Once transported across the membrane, the fatty acids are activated to acyl-CoAs by the ACSs Faa1p and Faa4p (Johnson et al., 1994). These studies in *E. coli* and yeast show that in addition to its integral role in lipid biosynthesis, ACS also has a role in fatty acid transport.

As well as being involved in the import of fatty acids into cells, ACSs are important in the intracellular movement of fatty acids. Many intracellular membranes act as barriers to the transfer of acyl-CoAs. Often, fatty acid transport involves the generation of free fatty acid and the subsequent reactivation to acyl-CoA after passage through the membrane (Ichihara et al., 1993; Igal et al., 1997). In animals, fatty acid import into the mitochondria involves both the activation to acyl-CoA at the outer membrane and a carnitine shuttle system at the inner membrane (Kerner and Hoppel, 2000). Therefore, as with fatty acid import in yeast and *E. coli*, fatty acid transport within the cell requires the activity of an ACS, which necessitates the presence of ACSs at distinct organellar locations. ACS activity has been localized in organelles within the plant cell, including oil bodies (Olsen and Lusk, 1994), peroxisomes (Gerbling and Gerhardt, 1987), mitochondria (Thomas et al., 1988), chloroplasts (Roughan and Slack, 1977; Joyard and Stumpf, 1981; Andrews and Keegstra, 1983), plastids (Fuhrmann et al., 1994), and microsomes (Ichihara et al., 1993). Although biochemical evidence for these ACS activities has been demonstrated, the enzymes have not been purified. There is little information about the total number of isozymes expressed and the contribution of each isozyme to overall fatty acid metabolism.

An ACS activity in the plastid outer envelope is responsible for the conversion of newly synthesized fatty acids (hydrolyzed from acyl-ACP) to fatty acyl-CoAs. Plastids are the site of essentially all de novo fatty acid synthesis. Two distinct pathways for the synthesis of glycerolipids and polyunsaturated fatty acids begin with the synthesis of 16:0 as an acyl-carrier protein (ACP) thioester (Browse and Somerville, 1991). Much of the 16:0-ACP is elongated to 18:0-ACP, which is then efficiently desaturated to 18:1-ACP, making 16:0-ACP and 18:1-ACP the primary products of plastid fatty acid synthesis. Fatty acids entering the prokaryotic pathway are not exported from the chloroplast. They are transferred from ACP by the action of glycerol-3-phosphate acyltransferase or lysophosphatidic acid acyltransferase and incorporated into chloroplastic membrane lipids (Ohlrogge and Browse, 1995). Alternatively, the fatty acid can be released from ACP by a thioesterase for subsequent export. Two principal types of thioesterase occur in plants, FatA and FatB. FatA, the major class, is specific for 18:1-ACP (Dormann et al., 1995), whereas the FatB class of thioesterases is specific for 16:0-ACP (Jones et al., 1995). Fatty acids cleaved from ACP by thioesterases are then targeted for export and can enter the eukaryotic pathway in the endoplasmic reticulum (ER). Before export from the chloroplast, these fatty acids must undergo conversion to acyl-CoAs by the outer envelope ACS.

Although the existence of a plastidial ACS has been known for many years, the details of fatty acid export from the plastid are not well understood. ACS was demonstrated as an activity of the chloroplast envelope (Roughan and Slack, 1977; Joyard and Stumpf, 1981) and later, more specifically of the outer envelope (Andrews and Keegstra, 1983; Block et al., 1983). Contact zones between the inner and outer envelope have been shown to exist (Douce and Joyard, 1990), and these zones may be sites of interaction where the thioesterase releases a fatty acid that is then activated to a CoA thioester by an ACS and subsequently exported. In Arabidopsis leaf mesophyll cells, 62% of the fatty acids synthesized in the chloroplasts are exported (Browse et al., 1986). In leaf cells of many other plants, more than 90% of fatty acids are exported to the ER with a return flux of lipids on the eukaryotic pathway providing the precursors needed for thylakoid membrane biogenesis (Browse and Somerville, 1991). In nonphotosynthetic tissues and developing seeds of all plants, 90% of the synthesized fatty acids are exported from the plastids (Browse et al., 1993). The dependence of fatty acid export on activation by ACS demonstrates the fundamental role a plastidial ACS plays in lipid metabolism.

Despite their significance in plant lipid synthesis, only limited biochemical data on ACSs are available. The cloning and characterization of ACSs has been recalcitrant in part due to their association with membranes. Fulda et al. (1997) reported cloning of five cDNAs from *Brassica napus* with homology to known yeast and *E. coli* ACSs, but only two of these cDNAs could be demonstrated to encode ACS activities after expression in *E. coli*. A third *B. napus* ACS has been described recently (Pongdontri and Hills, 2001). Our laboratory has focused on the cloning of ACSs from Arabidopsis. We identified and cloned a large family of long-chain ACSs (LACS) that has been summarized recently (Shockey et al., 2002). The cloning of this family has allowed us to perform genetic analyses of the LACSs. Here, we describe the identification and characterization of one gene, *LACS9*, that encodes a major chloroplast LACS.

RESULTS

Identification of a Potential Plastidial LACS Isoform

The isolation and cloning of nine Arabidopsis *LACS* genes are described in a companion paper (Shockey et al., 2002). Shockey et al. (2002) used

complementation in a yeast mutant as well as assays of recombinant enzymes to establish the cloned LACSs as functional ACS enzymes. Among these nine, we were interested in identifying a plastidial LACS isoform because it is required for acyl-lipid synthesis in all tissues of the plant. Previous biochemical studies localized plastidial LACS activity in the chloroplast outer envelope (Andrews and Keegstra, 1983; Block et al., 1983). Among other chloroplastic outer envelope proteins studied, most are not synthesized as higher M_r precursors and therefore do not contain a typical N-terminal transit peptide (Tranel et al., 1995). For this reason, we did not expect to identify a plastidial LACS isoform based on sequence analysis alone. Computer software programs, including PSORT (Nakai and Kanehisa, 1992) and ChloroP (Emanuelsson et al., 1999), were unsuccessful at identifying plastidial LACS isoforms (data not shown).

As an alternative approach to identifying a potential plastid-localized LACS, we analyzed RNA expression patterns to identify candidates with transcripts abundant in tissues active in de novo fatty acid synthesis. We reasoned that a plastidial LACS involved in the export of newly synthesized fatty acids for membrane and TAG synthesis would be expressed predominantly in young leaves and developing seeds. Initial RNA gel-blot analysis with gene-specific probes for the nine *LACS* isoforms indicated that expression patterns varied greatly among the tissues tested (data not shown). The transcript of one isoform, *LACS9*, was highly expressed in both siliques and young leaves. The results of the *LACS9* northern are shown in Figure 1. The tissues represented include flowers and buds, developing siliques, young leaves, older leaves, and roots. The *LACS9* probe hybridized to RNA from flowers and buds, and was more abundant in developing siliques. Siliques corresponding to 1 through 5 and 6 through 11 DAF contained the highest levels of *LACS9* transcript of all tissues examined. *LACS9* transcript was not detectable in mature seed (data not shown) and was present at low levels in older siliques (12–20 DAF), leaves from older plants (50 d), and roots. Although present to a lesser extent than in young, developing siliques, *LACS9* accumulated to modest levels in young leaves from 14- and 16-d-old plants. These results are consistent with an expression pattern we expect of a plastidial LACS involved in de novo fatty acid synthesis.

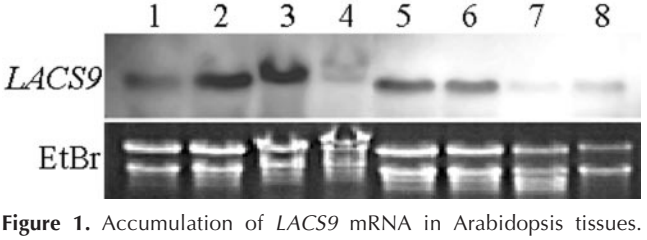

Figure 1. Accumulation of *LACS9* mRNA in Arabidopsis tissues. Total RNA (15 μg) was probed with a fragment of the *LACS9* transcript. Ethidium bromide staining of the major rRNA bands was used to confirm equal loading of total RNA. Lane 1, Flowers and buds; lane 2, 1- to 5-d after flowering (DAF) siliques; lane 3, 6- to 11-DAF siliques; lane 4, 12- to 20-DAF siliques; lane 5, primary leaves from 14-d old plants; lane 6, primary leaves from 16-d old plants; lane 7, leaves from 50-d old plants; lane 8, roots. *LACS9* transcript was not present in appreciable amounts in mature seed (data not shown).

Confirmation of the Plastidial Localization of LACS9

To further investigate the possibility that LACS9 was a plastidial isoform, we performed in vitro chloroplast import assays that provided a rapid and simple way to assess the association of LACS9 with chloroplast membranes (Bruce et al., 1994). Our assay included the small subunit (SSU) of Rubisco (a stromal-targeted protein; Olsen and Keegstra, 1992), a hydroperoxide lyase (LeHPL, a protein shown to be targeted to the outer envelope; Froehlich et al., 2001), and LACS9. Radiolabeled protein precursors were synthesized in vitro and incubated with intact pea (*Pisum sativum*) chloroplasts. Membranes from the chloroplasts were then repurified to remove excess precursor proteins and lysed in hypotonic buffer. The lysed chloroplasts were collected by centrifugation. Samples of the membrane fraction were washed in chaotropic buffers (2 M NaCl or 100 mM Na_2CO_3) to determine the strength of the association with membranes. Supernatant fractions were also recovered. Figure 2 summarizes the results of the import assays. The Rubisco SSU is targeted to the stroma of the chloroplast by a transit peptide that is cleaved after import (Froehlich et al., 2001). Figure 2 shows that labeled SSU (prSS) was targeted to the chloroplasts and processed to its mature form and was present in the soluble fraction after all treatments. LeHPL, a

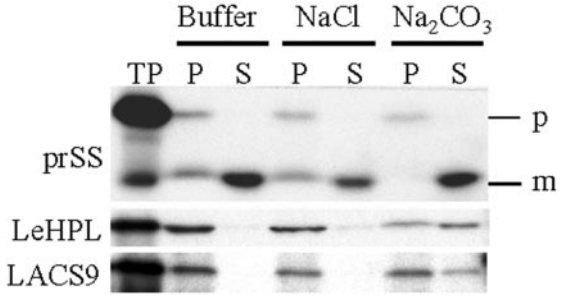

Figure 2. In vitro chloroplast import assays. ^{3}H-labeled Rubisco small subunit (prSS), LeHPL, and LACS9 were incubated with intact chloroplasts. The chloroplasts were repurified, lysed, and the membranes pelleted by centrifugation. After resuspension in lysis buffer, samples of the membrane fraction were washed either with the same lysis buffer, or NaCl or Na_2CO_3. Samples were analyzed by SDS-PAGE and fluorography. TP, Translated product; P, membrane fraction; S, soluble fraction; p, precursor; m, mature protein. Results shown are from one of three separate experiments that showed comparable results.

hydroperoxide lyase from tomato (*Lycopersicon esculentum*), has been shown to associate with the chloroplast outer envelope despite the lack of a typical transit peptide (Froehlich et al., 2001). In Figure 2, LeHPL associated with chloroplast membranes. Treatment of the membranes with lysis buffer or NaCl did not extract LeHPL. Only extraction with Na_2CO_3 began to dissociate LeHPL from the membranes, suggesting it is strongly associated with the membranes, as reported (Froehlich et al., 2001). LACS9 was also targeted to intact chloroplasts and was present only in the membrane fractions. Like LeHPL, the lysis buffer and NaCl treatments did not dissociate LACS9 from the membrane fraction of lysed chloroplasts, whereas sodium carbonate extracted a portion of LACS9 from chloroplast membranes. From these results, we conclude that LACS9 is strongly associated with chloroplast membranes. In addition, LACS9 does not appear to be proteolytically processed during plastidial targeting because the gel mobility of chloroplast-associated LACS9 was identical to that of the in vitro-translated product. The absence of processing is consistent with localization at the chloroplast envelope as opposed to localization in thylakoid membranes (Froehlich et al., 2001).

To provide further evidence that LACS9 is localized in the plastid envelope, we analyzed the transient expression of a LACS9-green fluorescent protein (GFP; at C terminus of LACS9) fusion in onion (*Allium cepa*) epidermal cells. The *LACS9-GFP* construct was simultaneously bombarded with a plastidial marker control vector containing an *ACP-DsRED* fusion. ACP is a soluble protein found in the plastid stroma. As shown in Figure 3, fluorescence from the LACS9-GFP fusion was strongly associated with the outer surface of organelles that the ACP-DsRED fluorescence confirmed as plastids. Outgrowths from these plastids are probably stromules (Kohler and Hanson, 2000) and these also contained LACS9-GFP. The patterns of fluorescence shown in Figure 3 indicate that LACS9 is a plastid envelope protein.

Identification of a *lacs9* Mutant by Reverse Genetics

To ascertain the contribution of LACS9 to normal plant growth and development, we initiated a search for an Arabidopsis T-DNA knockout mutant. A search of the T-DNA tagged populations available through the Arabidopsis Biological Resource Center (ABRC; Feldmann, 1991) was performed using a PCR-based screen with primers designed to either the 5′ or 3′ portions of *LACS9* in combination with T-DNA border primers. We generated a PCR band consistent with the presence of a T-DNA interrupting the *LACS9* coding region. Sequence analysis of the PCR product generated by using the T-DNA left border primer (LB) and 5′ gene-specific primer (P1) combination revealed the presence of a plant line containing a T-DNA insertional event in the third exon of *LACS9* (Fig. 4A). The sequence also showed that 21 bp of the T-DNA left border was truncated upon insertion. Other details on the nature of the insertional event were not investigated.

Seed pool CS2597 (ABRC) contained the plant with the T-DNA insertion in *LACS9* and this pool represented seed from 10 individual transformants. To identify mutant (*lacs9-1*) individuals, seed from pool CS2597 was surface sterilized and plated on germination medium containing kanamycin. After 10 d, 96 kanamycin-resistant individuals were transferred to soil. Genomic DNA was isolated from 12 pools containing eight plants each. The LB/P1 primer combination identified two pools with a *lacs9* mutant. Genomic DNA was then isolated from the 16 individual plants. To differentiate heterozygous from homozygous mutants, two PCR reactions were performed for each plant. First, P1/P2 primers were used to screen for the wild-type *LACS9* allele. Next, the LB/P1 primers were used to identify the presence of the mutant allele. From the 96 plants, we isolated one plant homozygous for a T-DNA insertion in *LACS9*, and one plant heterozygous at this locus. Seeds from the heterozygous plant were germinated on kanamycin plates to investigate the number of

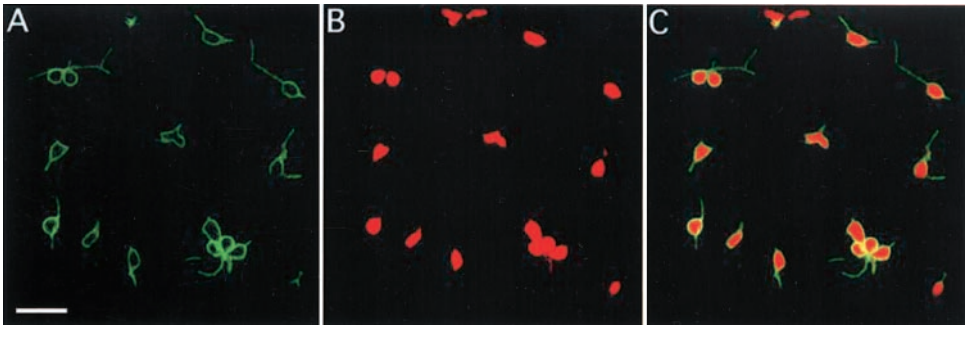

Figure 3. Transient expression of *LACS9-GFP* and *ACP-DsRED* in a bombarded onion epidermal cell. A, LACS9-GFP is localized to the periphery of organelles tentatively identified as plastids. B, ACP-DsRED fluorescence confirms the identification of plastids. C, A and B merged, showing that LACS9 expression is localized in the plastidial envelope and associated stromules. The bar represents 10 μm.

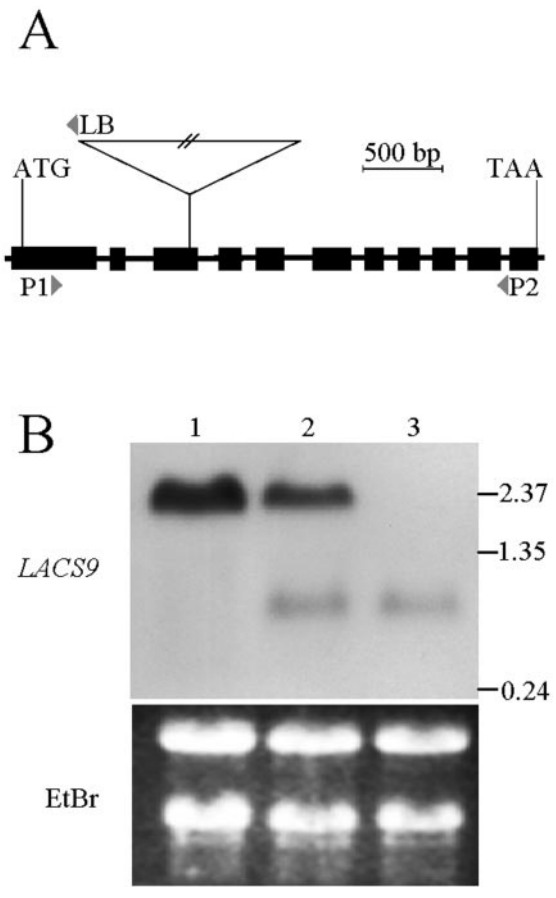

Figure 4. A, The structure and organization of the *LACS9* genomic sequence containing a T-DNA insertion. The *lacs9-1* knockout mutant contains a T-DNA insertion in the third exon, 1,020 bp from the start codon in the genomic DNA. The *LACS9* gene is located on chromosome I and is 3,324 bp in length (MIPS code At1g77590). B, Northern analysis of RNA isolated from the *lacs9-1* homozygous and heterozygous mutant plants compared with wild type. Total RNA (15 μg) from tissues of wild-type (lane 1), heterozygous (lane 2), and homozygous (lane 3) mutants was separated by electrophoresis and probed with a fragment of the *LACS9* cDNA. Ethidium bromide staining shows equal RNA loading.

T-DNA insertional events in *lacs9-1*. Of 471 seeds, 121 were kanamycin sensitive, which is a good fit to the 3:1 hypothesis for a single insertion ($\chi^2 = 0.033$; $P > 0.9$). To determine if the *lacs9-1* mutant had altered levels of LACS9 transcript in response to a T-DNA insertion in the open reading frame, total RNA was isolated from tissues of wild-type, heterozygous, and homozygous mutants and used for northern analysis (Fig. 4B). Wild-type plants expressed full-length *LACS9* transcript. The heterozygous mutants also expressed this full-length *LACS9* transcript. Both the heterozygous and homozygous plants contained a low level of a smaller M_r RNA that hybridized to the *LACS9* probe. This transcript is equal in size to a truncated *LACS9* transcript predicted to be produced by premature termination of *LACS9* transcription at or near the site of the T-DNA insertion (Fig. 4A). It can be inferred that any protein translated from this mutant transcript would not be functional because both the putative AMP-/ATP-binding domain and the proposed fatty acid substrate-binding pocket are located downstream of the insertion (Black et al., 1997; Black et al., 2000; Shockey et al., 2002). Based on this analysis, it is very likely that the *lacs9-1* line is a null for LACS9 activity.

Phenotypic Analysis of the *lacs9-1* Homozygous Knockout Mutant

The *lacs9-1* mutant was indistinguishable from wild-type controls in size and appearance when grown under normal culture conditions. To test for quantitative differences in plant growth, we grew wild-type and *lacs9-1* plants at 22°C under a 14:10 (light:dark) photoperiod. Changes in rosette fresh weight were measured by harvesting and weighing plants between 15 and 25 d after sowing. Figure 5 shows the resultant growth curve of wild-type versus *lacs9-1* plants. The relative growth rate of *lacs9-1* ($\omega^{-1} = 0.309 \pm 0.013$) was not significantly different from that of the wild type ($\omega^{-1} = 0.301 \pm 0.007$). The lack of variation in quantitative measurements of growth rate correlates with the observation that there were no outward phenotypic differences between wild-type and *lacs9-1* plants. Similar results were obtained when the plants were grown under 16:8 (light:dark) photoperiod (data not shown). In addition, no visible alterations in cellular ultrastructure or leaf anatomy were observed using transmission and scanning electron microscopy on leaves from 13- to 16-d-old plants (data not shown).

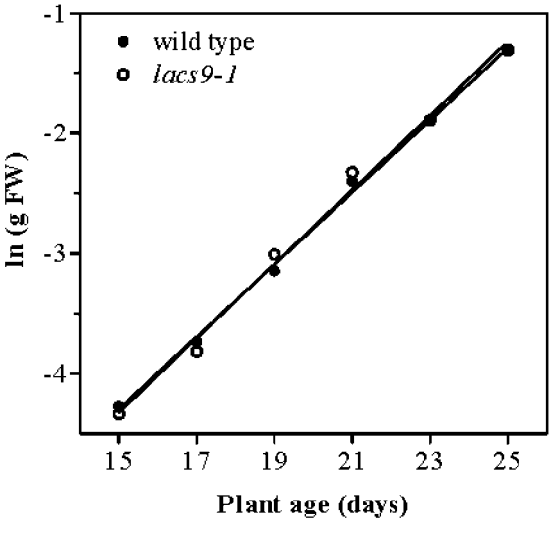

Figure 5. Growth curves for wild type and the *lacs9-1* plants grown at 22°C under 14:10 (light:dark) photoperiod. Growth measurements were made by taking the fresh weight of the aboveground portions of plants at the indicated intervals. The relative growth rate (ω^{-1}) for the wild type was 0.301 ± 0.007; for the mutant, it was 0.312 ± 0.013. Values shown are the means ± SE ($n = 10$).

The prokaryotic and eukaryotic pathways of lipid synthesis contribute almost equally to chloroplast membrane lipid production in Arabidopsis (Browse et al., 1986). The balance of fluxes through these pathways may be altered to alleviate a block in one of the pathways (Browse and Somerville, 1991). For example, the *fad2* mutant defective in the ER oleoyl-phosphatidylcholine desaturase exhibits a decreased flux through the eukaryotic pathway and a compensating increase in chloroplast lipid synthesis by the prokaryotic pathway (Miquel and Browse, 1992). In principle, a decrease in plastidial fatty acid export resulting from the *lacs9* mutation might be ameliorated by reduced flux of lipid from the ER back to the chloroplast on the eukaryotic pathway, and a corresponding increase in chloroplast lipid synthesis via the prokaryotic pathway. Such a shift in fluxes through the two pathways is predicted to increase the levels of 16:3 (a specific product of the prokaryotic pathway) in monogalactosyl diacylglycerol and in the overall leaf fatty acid profile. Both of these predicted changes were observed in *fad2* plants (Miquel and Browse, 1992). We extracted leaf lipids from wild-type and *lacs9-1* plants and analyzed the fatty acid compositions of individual lipids separated by thin-layer chromatography (TLC). There was no significant difference between wild type and mutant in the proportion of 16:3 in total leaf extracts or in purified monogalactosyl diacylglycerol (data not shown), nor did we discern any other changes that could be interpreted in terms of reduced transfer of acyl groups between the chloroplast and ER.

Because northern analysis showed that the *LACS9* transcript was more abundant in developing siliques than young leaves (Fig. 1), we monitored the accumulation of fatty acids in developing siliques. Significant changes in the amount of fatty acid methyl esters (FAMEs) were not observed in developing siliques from wild type and *lacs9-1*. In addition to very similar patterns of lipid accumulation, mutant and wild-type seeds were indistinguishable in size and appearance. Furthermore, the fresh weight and total FAMEs present in mature seed was not significantly different between wild type and *lacs9-1* (data not shown).

LACS9 Contribution to Plastidial LACS Activity

The absence of any detectable phenotype in the *lacs9-1* mutant and the expectation that a defect in a major chloroplast LACS would result in a pronounced phenotype raised the possibility that LACS9 is a minor LACS isozyme with minimal contribution to overall chloroplast LACS activity. However, the relative abundance of *LACS9* transcript and our demonstration that LACS9 is an active LACS (Shockey et al., 2002) argue against such a conclusion. To directly measure the contribution of the LACS9 isoform to chloroplast LACS activity, we performed in vitro LACS assays on chloroplasts isolated from leaves of 19-d-old wild-type and *lacs9-1* plants. Isolated chloroplasts were first assayed for LACS activity in hypotonic media using 1-[^{14}C]18:1 or 1-[^{14}C]16:0 as a substrate. Control assays lacking CoA, or in which boiled chloroplasts were used, demonstrated negligible activity (data not shown). Figure 6 shows that the LACS activity of wild-type chloroplasts averaged 9.3 nmol fatty acyl-CoA min^{-1} mg^{-1} chlorophyll (Chl). In contrast, LACS activity of *lacs9-1* chloroplasts averaged 0.98 nmol min^{-1} mg^{-1} Chl, or 10% of the wild-type activity. Similar results were obtained when the assay was performed in osmotic buffer to prevent chloroplast lysis. This result is consistent with the chloroplast LACS being localized in the envelope with access to fatty acid substrates available in the external medium. Taken together, these results suggest that LACS9 is a major contributor to chloroplastic LACS activity.

DISCUSSION

ACS isozymes catalyze important steps in many pathways of fatty acid and lipid metabolism. To understand the biochemistry and biology of these isozymes in plants, we have used genomics approaches, together with complementation and enzyme assays, to definitively identify nine LACS genes in Arabidopsis (Shockey et al., 2002). We were particularly interested in identifying a plastidial isoform because of its key role in cellular lipid synthesis. However, identifying a plastidial LACS from the nine isoforms was not straightforward. The mode of targeting of plastidial LACS isozyme(s) is currently unknown. The insertion of other proteins into the chloroplast outer envelope membrane is thought to

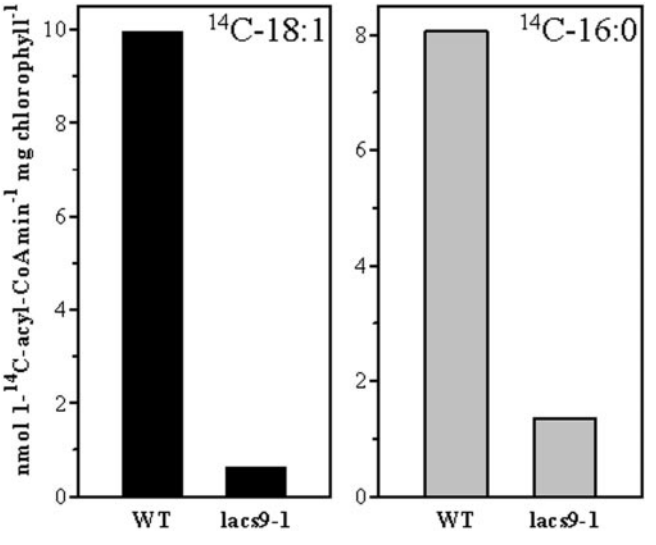

Figure 6. In vitro LACS assay on isolated chloroplasts from wild-type and *lacs9-1* plants. Intact chloroplasts were isolated and then assayed with 1-[^{14}C]18:1 or 1-[^{14}C]16:0 substrates in hypotonic media (see "Materials and Methods" for details).

occur by one of two routes (Keegstra and Cline, 1999). Proteins that lack a typical transit peptide use a pathway that does not require ATP or surface-exposed receptors (Froehlich et al., 2001). In the second pathway, targeting of proteins to the outer envelope is directed by an N-terminal transit peptide that is subsequently cleaved. This pathway requires components of the general import apparatus (Keegstra and Cline, 1999). We were unable to identify potential transit peptides in the predicted protein sequences of the nine Arabidopsis LACS. Instead, we relied on examination of RNA expression profiles to narrow our search. The expression pattern of *LACS9* suggested that it might represent a plastidial isoform participating in fatty acid export. The *LACS9* transcript was most abundant in developing siliques and young leaves (Fig. 1), which are tissues actively synthesizing triacylglycerol or membrane lipids, respectively.

To confirm that the LACS9 isoform was targeted to plastid membranes, we performed in vitro chloroplast import assays. Radiolabeled LACS9 protein strongly associates with pea chloroplast membranes (Fig. 2). A sodium carbonate treatment did not extract LACS9 from the membranes to any great extent. Another enzyme targeted to the outer envelope of the chloroplast, LeHPL, exhibited a similar pattern, suggesting that both LeHPL and LACS9 are intrinsic membrane proteins (Froehlich et al., 2001). The assay also indicated that the LACS9 protein was not proteolytically processed upon association with chloroplast membranes. This is consistent with the targeting of LACS9 to the outer envelope of the chloroplast via the ATP-independent pathway (Froehlich et al., 2001). Further evidence that LACS9 is localized in plastidial envelope membranes was obtained by transiently expressing a LACS9-GFP fusion protein. Figure 3 shows GFP fluorescence localized in the envelope of onion epidermal cell plastids. Although the import assays and GFP expression gave strong indications that LACS9 was a plastid isoform, perhaps the most convincing evidence of plastidial localization came from ACS assays of chloroplasts isolated from wild-type and *lacs9-1* mutant plants. Chloroplasts isolated from *lacs9-1* exhibited only 10% of the assayable LACS activity found in wild-type chloroplasts using 1-[^{14}C]18:1 or 1-[^{14}C]16:0 as a substrate (Fig. 6).

Assays of ACS activity in purified chloroplast preparations from *lacs9-1* and wild-type plants indicate that the knockout mutant has a low residual activity. However, *lacs9-1* mutant plants were indistinguishable from wild type in appearance, growth rate, and the rate and extent of storage lipid accumulation in developing siliques. Also, we were unable to detect any alterations in leaf fatty acid or lipid composition that might indicate changes associated with decreased export of acyl groups from their site of synthesis in the chloroplast. These observations indicate that the residual LACS activity measured in *lacs9-1* chloroplasts must be sufficient to support cellular lipid synthesis and plant growth. Alternatively, it might be suggested that the activities measured in our assays reflect a minor isozyme encoded by *LACS9* and that the major isozyme remained latent or was inhibited during tissue homogenization or chloroplast purification. To address these questions, we compared our assay data with predicted rates of chloroplast fatty acid export. Assays on isolated chloroplast preparations from four plant species showed a range in fatty acid synthesis rates from 1,400 to 3,300 nmol carbon h^{-1} mg^{-1} Chl (Heinz and Roughan, 1983) and isolated spinach chloroplasts exhibit rates of approximately 4,000 nmol C h^{-1} mg^{-1} Chl (Roughan and Slack, 1977). Estimates of in vivo rates of fatty acid synthesis measured in intact leaves are lower, but also show a substantial range depending on leaf age, light conditions, and other factors; estimates from 700 to 2,500 nmol C h^{-1} mg^{-1} Chl have been reported (Murphy and Leech, 1977; Browse et al., 1981; Bao et al., 2000). A substantial proportion of newly synthesized fatty acids enter the prokaryotic pathway in leaf chloroplasts and only 62% exit the chloroplast as acyl-CoAs to supply the eukaryotic pathway (Browse et al., 1986). Thus, the in vivo rates of fatty acid synthesis indicate rates of acyl-CoA synthesis at the chloroplast envelope of 490 to 1,550 nmol C h^{-1} mg^{-1} Chl. The average rate of ACS activity in our preparations of *lacs9-1* chloroplasts was 0.98 nmol fatty acyl-CoA min^{-1} mg^{-1} Chl, which corresponds to approximately 880 nmol C h^{-1} mg^{-1} Chl. Given the ambiguities in these estimates and comparisons, it is reasonable to conclude that the rates of chloroplast LACS activity in *lacs9-1* leaves may well be sufficient to support cellular membrane biogenesis. Certainly, there is no need to invoke an isozyme that was cryptic to our assays; therefore, we conclude that *LACS9* does encode a major chloroplast isozyme accounting for nearly 90% of the total chloroplast LACS activity. The rates of LACS activity measured in wild-type chloroplasts are more than 5-fold higher than even the highest rates calculated for fatty acid export from the chloroplast. It is not clear why such an excess of activity is maintained.

Although *LACS9* encodes a major chloroplast LACS isozyme, it is clear that one or more other genes encode isozymes that are functionally redundant and support acyl-CoA synthesis and export from the chloroplast in the *lacs9-1* knockout mutant. Currently, we do not know the gene (or genes) encoding the remaining chloroplast activity. One likely candidate is the *LACS8* gene that is homologous to *LACS9* (Shockey et al., 2002). The predicted amino acid sequence of LACS8 is 78% similar to LACS9, whereas pair-wise comparisons between LACS9 and the remaining seven LACS proteins characterized from Arabidopsis yielded similarities in the range of 45% to 50%. Preliminary results from in vitro chloroplast import assays indicate that the LACS8 protein

may be targeted by the chloroplast membranes as was LACS9, but these experiments need to be extended and confirmed by cell biology approaches such as GFP fusion studies. Unfortunately, we have not yet been able to identify a T-DNA insertion at the *LACS8* locus among the currently available T-DNA populations. Therefore, it may be necessary to use other reverse genetics approaches (Wesley et al., 2001) to generate lines deficient in LACS8 activity either in a wild-type or mutant *lacs9-1* genetic background. Whether or not *LACS8* encodes the remaining chloroplast activity, it is clear that identification and characterization of other plastidial LACSs will provide information on why multiple plastid isozymes are present and what the role of each isozyme is in lipid synthesis in different cell types and organs of the plant.

MATERIALS AND METHODS

Plant Growth and Mutant Isolation

The wild-type line of Arabidopsis used in this study is the Wassilewskija ecotype. The *lacs9-1* knockout mutant (ABRC seed stock CS2597) is in the Wassilewskija background. Plants were germinated and grown on a commercial potting mixture at 22°C under illumination of fluorescent lights (175 μmol m^{-2} s^{-1}) under a 16:8 (light:dark) photoperiod unless otherwise noted. For measurement of growth rate, samples of 10 randomized plants were individually harvested at 2-d intervals, and the aerial portions were weighed.

The Arabidopsis T-DNA-tagged lines available through the ABRC (Feldmann, 1991) were screened by doing PCR on pooled DNA with the T-DNA left border primer (KFLB; 5′-TGCACTCGAAATCAGCCAATTTTAGACAA-3′) in combination with the 5′ *LACS9* primer (P1; 5′-GAAAGTTAAACTCAATTCCTCCTGCGATCA-3′) or the 3′ *LACS9* primer (P2; 5′-GCATATAACTTGGTGAGATCTTCAGAGAATT-3′). The DNA pools were screened according to the protocols suggested by the Arabidopsis knockout facility (http://www.biotech.wisc.edu/Arabidopsis/). Seeds were surface sterilized in 20% (v/v) bleach + 0.1% (v/v) SDS for 20 min and rinsed in sterile water. Sterilized seed suspended in 0.1% (w/v) agarose were germinated on medium containing 4.3 g L^{-1} Murashige and Skoog salts (Murashige and Skoog, 1962), pH 5.8; 1% (w/v) Suc; 0.35% (w/v) Phytagel (Sigma, St. Louis); and 75 mg L^{-1} kanamycin. After 10 d, resistant plants were removed and transferred to soil.

Northern Analysis

Total RNA from seeds and silique tissues was isolated using the protocol of Vicient and Delseny (1999). All other RNA was isolated according to the Trizol protocol (Sigma). RNA was separated on a 1% (w/v) agarose gel in formaldehyde and transferred to nylon membrane overnight in 10× SSC (1.5 M NaCl and 0.15 M Na-citrate, pH 7.0). Digoxygenin-labeled probes were synthesized with a PCR-labeling kit (Roche Applied Science, Indianapolis) and blots were hybridized and washed at high stringency according to the manufacturer's protocol. The *LACS9* probe (bp 1–443 in the cDNA) shares less than 60% identity with the closest homolog, *LACS8* (see Shockey and Browse, 2002).

In Vitro Chloroplast Import Assays

Pea (*Pisum sativum* var Span) seeds (Crites-Moscow Growers, Inc., Moscow, ID) were germinated in vermiculite and grown under a 16:8 (light:dark) photoperiod. Chloroplasts were isolated from 9- to 10-d-old pea seedlings essentially as described (Bruce et al., 1994). Intact chloroplasts were recovered and resuspended in import buffer (330 mM sorbitol and 50 mM HEPES/KOH, pH 8.0) at 1 mg mL^{-1} Chl.

The plasmid containing prSS was a gift from Dr. Ken Keegstra (Michigan State University, East Lansing; Olsen and Keegstra, 1992) and the plasmid containing LeHPL was a gift from Dr. Gregg Howe (Michigan Stage University). The pJAS25 plasmid contains a full-length cDNA of *LACS9* in pET24d (Invitrogen, Carlsbad, CA). The in vitro transcription/translation reactions were performed by using the TNT-coupled wheat germ lysate system (Promega, Madison, WI) with [^{3}H]Leu (NEN, Boston). Import reactions (adapted from Bruce et al., 1994) received 3×10^6 dpm of translation product after the addition of intact chloroplasts (150 μg Chl) in 450 μL. Reactions were incubated for 30 min at 25°C in the light. Intact chloroplasts were recovered by sedimentation through a 40% (v/v) Percoll cushion. Pellets were resuspended in lysis buffer (25 mM HEPES-KOH, pH 8.0; and 4 mM $MgCl_2$), incubated for 20 min on ice, divided into three equal portions, and pelleted at 100,000*g* for 30 min. Pellets were resuspended in either lysis buffer, 2 M NaCl, or 100 mM Na_2CO_3 (Tranel et al., 1995). After ultracentrifugation at 100,000*g* for 30 min, total membrane and soluble fractions were obtained. Protein was precipitated from the soluble fractions with 10% (v/v) trichloroacetic acid. All fractions were analyzed by SDS-PAGE (Laemmli, 1970) and fluorography. Luciferase was used as a negative control (Promega) and this protein did not associate with the chloroplasts repurified from the import reactions (data not shown).

Transient Expression and Localization Studies of LACS9 in Onion Epidermal Cells

The *LACS9* cDNA sequence was cloned into the transient expression vector pEZS-LN (a gift from Gert-Jan de Boer and Dave Ehrhardt, Carnegie Institution of Washington) to create pJAS33 (5′-*CaMV35S-LACS9-GFP*-3′). A fusion of ACP with DsRED (Matz et al., 1999) was used as a positive control for plastid localization (pGJ102R). For transient expression in onion epidermal cells, 25 μL of gold particles (1-μm diameter, 60 mg mL^{-1} in ethanol) were washed in water before being mixed with 5 μg of plasmid DNA. After addition of 50 μL of 2.5 M $CaCl_2$ and 25 μL of 0.1 M spermidine, the DNA was precipitated on the gold particles at room temperature for 3 min with continuous shaking. The gold pellet was washed once in 100% (v/v) ethanol before being resuspended in 25 μL of 100% (v/v) ethanol. Aliquots of gold were spotted on macrocarriers and used to transform onion epidermal cells at 1,300 psi using a PDS 1000HE biolistic device (Bio-Rad, Hercules, CA). The bombarded tissue was mounted on microscope coverslips and immersed in 1× Murashige and Skoog medium 12 to 24 h after transformation. Localization was examined using confocal microscopy as described by Cutler et al. with small modifications (Cutler and Ehrhardt, 2000). To eliminate fluorescence in the green channel due to expression of ACP-DsRED, a preset value determined empirically by examining cells expressing ACP-DsRED was subtracted from the signal obtained in the green channel using the Lasersharp software during confocal imaging. Three-dimensional images of cells were reconstructed by importing the data sets into NIH image or ImageJ software, available from the National Institutes of Health. To merge data sets from the different fluorescent channels, the images were imported into the appropriate color channels in Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Fatty Acid and Lipid Analysis

FAME analysis of leaf tissues was done essentially as described (Miquel and Browse, 1992). The lipid content of leaf tissues was determined by extraction as described (Browse et al., 1986). Extracted lipids were separated by TLC on $(NH_4)_2SO_4$-impregnated silica plates with the solvent system of acetone:benzene:water (30:10:2.7 [v/v]; Khan and Williams, 1977). FAME analysis of individual lipids scraped in silica gel from these plates was carried and as described above. For silique analysis, axillary and secondary inflorescences were removed as they appeared. At 42 d, intact siliques were harvested. The number of siliques removed per sample was determined by dividing the total number of siliques by 10. The samples were removed (excluding the oldest and two youngest samples) and methylated with 17:0 free fatty acid as an internal standard.

In Vitro ACS Assay

Chloroplasts (equivalent to 20 μg Chl) were isolated from 19-d-old plants as described above and were added to assay buffer (100 mM Bis-Tris-

propane, pH 7.6; 10 mM $MgCl_2$; 5 mM ATP; 0.5 mM CoA; 2.5 mM dithiothreitol; and 1 mM 1-[^{14}C]oleic acid [1.96 GBq mmol^{-1}, NEN]) in 100 μL. Assays were terminated by addition of 100 μL of acetic acid:isopropanol (10:90 [v/v]). Reactions were extracted twice with 900 μL of water-saturated hexane, with vigorous vortexing and centrifugation at 5,000*g* for 5 min. After the second extraction, 100 μL of aqueous phase was added to 10 mL of scintillation cocktail (BCS:water, 90:10 [v/v]; Amersham, Piscataway, NJ). The products of the assay were analyzed by TLC in butanol:acetic acid:water (5:2:3 [v/v]). More than 99% of the label was recovered in a band corresponding to an acyl-CoA standard (data not shown). Leaves from wild type and *lacs9-1* do not contain significantly different amounts of Chl on a fresh weight basis (1.90 ± 0.02 mg Chl g^{-1} fresh weight in each).

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